be involved in the quality control of glycoprotein folding in the ER (Parker *et al.*, supra; Fernandez *et al.*, supra; M. C. Sousa and A. J. Parodi, The interaction of UDP-Glc:Glycoprotein Glucosyl transferase with the acceptor glycoprotein, Cellular and Molecular Biology 42: 609-616 (1996); Sousa MC and Parodi AJ., The molecular basis for the recognition of mis-folded glycoproteins by the UDP-Glc: Glycoprotein Glucosyl transferase, EMBO J 14: 4196-4203 (1995)). UGGT participates together with lectin-like chaperones that recognize monoglucosylated oligosaccharides in the control mechanism by which cells only allow passage of properly folded glycoproteins to the Golgi apparatus (Labriola *et al.*, J. Cell Biol. 130(4):771-9 (1995)).

Cycles of transient interaction with UGGT, each resulting in reglucosylation of attached oligosaccharides, is believed to facilitate interaction between unfolded glycoproteins and calnexin and ensure the intracellular retention of improperly folded glycoproteins in the ER. Calnexin binds to glucose residues which are exposed on the N-linked sugar chains of membrane proteins.

It has been shown that UGGT requires millimolar calcium concentrations for optimal activity (Trombetta and Parodi, 1992). In cells expressing wild type α1 antitrypsin, treatment with thapsigargin retards or prevents the secretion of the protein (Kuznetsov et al., 1993; Lodish and Kong, 1990). This is apparently due to stable association of the newly synthesized α1-antitrypsin with UGGT in the endoplasmic reticulum when calcium levels in the ER are reduced (Choudhury et al., 1997). It has also been shown that lowering ER calcium through application of thapsigargin or calcium ionophores retards the exit of numerous wild type proteins from the ER and increases their rate of degradation (Wilkstrom and Lodish, 1993; Sudbeck et al., 1997; van Weering et al. 1998; Clark et al., 1994; Wong et al., 1993; Wileman et al., 1991; Lodish et al., 1992; Lodish and Kong, 1990). While not wishing to be bound by any theory, it may be the case that if the UGGT enzyme is denied calcium, it binds tightly to its substrates (i.e. newly synthesized glycoproteins) but is unable to release them, perhaps because successful completion of the glucose transfer step is required to effect release. Of course retention of misfolded proteins may occur through any of a number of other mechanisms.

It is interesting to speculate why, in the case of α1-antitrypsin, thapsigargin retards protein exit from the ER, whereas in the case of ΔF508 CFTR exit from the ER is stimulated by this drug (see Examples 1-8). Without wishing to be bound by any theory, we propose that in cells expressing a mutant protein that is incapable of proper folding, mis-folded protein is present in the ER in quantities which constitute a large molar excess

over the resident quantity of UGGT. Under normal circumstances, the mis-folded protein binds to UGGT, undergoes addition of a glucose residue and is rapidly released (Hammond and Helenius, 1995). The glucosylated protein is retained in the ER via interactions with calnexin, and a sufficient pool of UGGT is available to interact with mis-folded proteins that have lost their glucose tag. When ER calcium is depleted, each molecule of UGGT becomes stably complexed with a mis-folded protein, and thus unavailable to interact with the remaining mis-folded proteins in the ER. Since the mis-folded proteins are present in large molar excess over the UGGT, the excess mis-folded protein is free to escape the UGGT-mediated quality control system and to exit the ER. In contrast, in cells that do not express a mutant mis-folded protein, we hypothesize that UGGT exists in large molar excess over its potential substrates. Thus, when ER calcium is depleted, UGGT may act as a sink that can bind up newly synthesized proteins that have not completed their folding. Consequently, the bulk of newly synthesized proteins are retained in the ER.

H. <u>Release of Mis-folded ΔF508 CFTR Protein From the ER.</u>

We have developed a novel strategy that releases mis-folded ΔF508 CFTR protein from the ER and allows it to be functionally expressed at the cell surface. While not wishing to be bound by any theory, it is believed that retention of mis-folded membrane proteins in the ER is dependent upon interactions with ER resident chaperone proteins. Biochemical characterization of chaperone activity reveals that optimal functioning of several of these proteins requires calcium concentrations in the millimolar range (S.K. Nigam, A.L. Goldberg, S. Ho, M.F. Rohde, K.T. Bush, M.Y. Sherman, *J. Biol. Chem.* 269,1744, 1994; S.E. Trombetta, A.J. Parodi, *J. Biol. Chem.* 267, 9236, 1992). Mobilization of sequestered ER Ca^{2+} stores with agents such as the ER Ca^{2+} pump inhibitor thapsigargin dramatically reduces the ER lumenal calcium concentration (M. Montero, J. Alvarez, W.J.J. Scheenen, R. Rizzuto, J. Meldolesi, T., Pozzan, *J. Cell Biol.* 139, 601, 1997). While not wishing to be bound by any theory, we postulate that exposing cells to thapsigargin might interfere with the capacity of chaperones to mediate the ER retention of mis-folded proteins and that depleting ER Ca^{2+} stores with thapsigargin would allow the mis-folded ΔF508 CFTR protein to "escape" from the ER and potentially reach the cell surface, where it would be able to function as a chloride channel and correct the CF defect.

As described in the Examples, we have shown that treatment of CF airway epithelial cells with thapsigargin, which reduces the calcium concentration in the ER lumen, leads to functional expression of the ΔF508-CFTR protein at the cell surface as

revealed by electrophysiologic and immunofluorescence analysis. In addition, we have shown that treatment with thapsigargin can induce reversal of a phenotypic defect in a mouse model for cystic fibrosis (CF mice). The dose of thapsigargin employed in these studies appears to be tolerable and induces an effect whose magnitude is probably sufficient to produce clinically significant improvements in airway epithelial function in cystic fibrosis patients.

Finally, it must be noted that the mechanism through which calcium pump inhibitors effect the release of ΔF508 CFTR from the ER may not be related directly to the calcium requirements of ER chaperone machinery. It is possible, for example, that depletion of calcium from the ER lumen is sufficient to facilitate the spontaneous folding of the ΔF508 CFTR protein, permitting it to acquire a stable conformation and bypass chaperone retention. In either case, it is clear that calcium pump inhibition is sufficient to release a cohort of ER-retained ΔF508 CFTR to the cell surface, where it can function appropriately (see Examples 1-8).

I. Rhinosinusitis and *CFTR* Mutations

Rhinosinusitis, or inflammation of the sinus epithelium, is an extremely common condition which can be divided into several subtypes including acute, recurrent acute, subacute, and chronic based typically on patient history and physical examination. The persistent form, chronic rhinosinusitis (CRS), affects approximately 14% of the U.S. population and is almost invariably present in patients with CF. A case-control study in which DNA of CRS patients (individuals with more than 8 weeks of nasal or sinus symptoms or with a history of at least 4 episodes of recurrent symptoms of greater than three weeks' duration in the prior 12 months) and controls was typed for 16 mutations that account for 85% of CF alleles in the general population and also tested for the presence of additional mutations and variants revealed that the proportion of CRS patients who were found to have a CF mutation in one of their copies of the CF gene (7%) was significantly higher than in the control group (2%) (Wang, X., *et al.* "Mutation in the Gene Responsible for Cystic Fibrosis and Predisposition to Chronic Rhinosinusitis in the General Population", *JAMA*, Vol. 284, No. 14, 2000). Approximately 90% of the patients with a CF mutation carried the ΔF508 allele. In addition, most of the CF carriers with CRS had variants in their other *CFTR* gene. In particular, the M470V variant was found in 9 of the 10 CRS patients with a CF mutation, and in 8 of these patients the M470V variant was in the gene that did not carry a CF-causing mutation. The variant with valine at amino acid position 470 has reduced chloride channel activity compared with that having methionine